Experimental Neurology 287 (2017) 288–297

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Cellular and network-level adaptations to in utero methadone exposure along the ventral respiratory column in the neonate rat

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ARTICLE INFO

Article history:
Received 16 December 2015
Received in revised form 17 March 2016
Accepted 18 March 2016
Available online 20 March 2016

Keywords:
ventrolateral medulla
methadone
neonatal abstinence syndrome
tolerance
Ca^{2+} imaging
in vitro
hypomyelination

ABSTRACT

Neonatal abstinence syndrome (NAS) occurs in babies chronically exposed to opioids during pregnancy. NAS shares features with opioid withdrawal symptoms seen in adults, including autonomic dysregulation. Here, the effect of low-dose in utero methadone (MTD) exposure on respiration-modulated networks along the ventral respiratory column (VRC) in ventrolateral medulla was investigated in the neonate Sprague–Dawley rat. MTD was administered via drinking water (3 mg/kg/day in drinking water of the mother E7-E21). Lower expression levels of myelin-associated proteins phosphorylated axonal neurofilament subunit H (pNFH), 2′,3′-Cyclicnucleotide 3′-phosphodiesterase (CNPase) and myelin basic protein (MBP), in MTD-exposed pups compared to controls at P3, P6 and P10 indicated MTD transport across the placenta. We investigated whether in utero MTD exposure led to network-level excitability changes consistent with tolerance, and also probed for changes in endogenous opioid modulation of respiratory networks. To this end, high-speed (45.5 Hz) optical recordings of respiratory network activity in control and MTD-exposed neonate (P0-P2) pups before and during administration of the μ-opioid receptor antagonist naloxone (NAL; 10 μM) were carried out. Spike rate was estimated from optical traces via deconvolution, and coupling between all neuron pairs in recorded networks was quantified using the normalized transfer entropy (NTE). Recordings of local networks along the VRC, together with recordings of respiratory output from ventral root C1 did not reveal changes in respiratory activity at the system level, but cellular and network changes in MTD-exposed pups were consistent with the development of opioid tolerance. MTD-exposed pups were found to have **i.** higher neuronal firing rates; **ii.** higher covariance between neuronal activity and motor output; **iii.** more bidirectionally and unidirectionally coupled neurons, and fewer uncoupled neurons; **iv.** stronger coupling and shorter integration times between network constituents. The μ-opioid receptor antagonist NAL did not alter system-level function. The correlation between the activity of neurons caudal to −400 μm and motor output was significantly reduced in control animals following NAL. In both control and MTD-exposed pups, the relative number of neurons whose correlation with motor output increased following NAL followed a rostrocaudal gradient along the VRC, with fewer neurons caudally, and more neurons rostrally. The up-regulation of coupling strength, firing rate and coefficient of variation between neurons and motor output following in utero opioid exposure suggests that these networks may contribute to NAS in infants born to opioid-dependent mothers.

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1. Introduction

In the last decade, the number of babies born to mothers addicted to opioids has increased 5-fold in the US (Patrick et al., 2012). The impact of in utero exposure occurs over short and long time-scales, and remains poorly understood. Shortly after birth, babies exhibit symptoms consistent with neonatal abstinence syndrome (NAS). These include sleeplessness, tremor, convulsions, sweating, hyperthermia, excessive yawning, rapid breathing, regurgitation, and loose stools (Finnegan, 1988). These acute symptoms, which are qualitatively similar to opioid withdrawal in adults, are treated with morphine and/or clonidine on a tapered dosage requiring prolonged hospital stays (Siu and Robinson, 2014). In addition to the acute symptoms associated with NAS, opioids regulate oligodendrogenesis in utero (Knapp et al., 2001). Thus in addition to NAS, in utero opioid exposure is associated with hypomyelination (Walhovd et al., 2010), and developmental delays over the course of childhood (Nygaard et al., 2015). The association between opioid exposure and hypomyelination make it desirable to find alternatives to morphine for symptom management in NAS. To this end, it is important to better understand how in utero opioid exposure alters cellular and network function in networks that contribute to NAS.

The complications that ensue from abrupt cessation of opioid exposure arise out of the action of opioids over multiple time-scales. μ-Opioid

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http://dx.doi.org/10.1016/j.expneurol.2016.03.020
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receptors (MORs) are G-protein coupled receptors that activate and regulate multiple second-messenger pathways (Williams et al., 2001). These signaling cascades give rise to acute effects that include depression of neuronal excitability via adenylyl cyclase inhibition; potassium conductance activation; calcium conductance inhibition; and the inhibition of transmitter release (Connor and Christie, 1999). Prolonged exposure to opioids gives rise to homeostatic restoration of neuronal excitability via desensitization, and, over longer time-scales, increase in opioid tolerance (Williams et al., 2013). The temporally overlapping processes of desensitization and tolerance are mediated by adaptive processes that directly reduce opioid receptor response, and counter-adaptive processes involving changes in non-opioid signaling pathways that oppose the action of opioids (Waldhoer et al., 2004).

These processes are understood as an adaptive response to the chronic depressive effect of opioids, arising out of the homeostatic regulation of neuronal and network excitability. This new equilibrium is disrupted following abrupt cessation of opioid intake, such that the adaptive responses associated with the development of tolerance now give rise to a rapid transition to hyperexcitability of opioid-modulated networks (Christie, 2008), and contribute to the dyspnea, hyperalgesia, dysphoria, and gut hypermotility associated with opioid withdrawal symptoms in adults. These processes may account for the same symptoms in NAS infants.

The role of opioid-sensitive cardiovascular and respiratory regulatory networks in ventrolateral medulla in withdrawal remains poorly understood. These networks are distributed along the ventral respiratory column (VRC) in ventrolateral medulla, and preparations that isolate these networks have been used to investigate opioid-induced respiratory depression (Mellen et al., 2003; Takeda et al., 2001). Thus it is plausible that adaptation processes in these networks might in part account for the autonomic dysregulation observed in NAS (Wolff and Perez-Montejano, 2014). Because survival depends on robust, adaptive respiratory network function at birth, prolonged opioid-induced depression in utero would require both cellular and network-level changes to compensate for opioid-induced depression. The optical recording methods used here provide little information about the biophysical mechanisms of such adaptations. Nonetheless, these methods allow us to simultaneously monitor cellular activity and network interactions. If opioids induce cellular and network-level counteradaptations to chronic opioid-induced depression, we hypothesize that these changes will be reflected in the cellular and network parameters that can be monitored via optical recordings.

In this study, these networks were exposed for optical recording in the sagittaly section rat hindbrain (SSRH) preparation (Mellen, 2008; Mellen and Funk, 2013), recorded at sampling rates high enough to permit inference of spike rate and coupling relations (Gourevitch and Mellen, 2014). Pups born to dams exposed to low, orally administered doses of methadone (MTD) were compared to control pups under control conditions, and following administration of the opioid receptor antagonist naloxone (NAL). System-level, network-level and cellular changes consistent with adaptation to chronic opioid exposure and the development of opioid tolerance were detected. These findings suggest that dysregulation of networks along the VRC may contribute to NAS.

2. Methods

2.1. Opioid preconditioning

Female Sprague–Dawley rats were housed with a male rat for 3–6 days, and were checked every morning for a plug to confirm insemination. Once a female was deemed to be pregnant, the male rat was removed from the cage, and, according to Drug Enforcement Administration-approved protocols, dams were maintained on methadone from E7 onwards. Based on normal water intake levels in pregnant rats (40 ml/day) (Atherton et al., 1982), in order to achieve a target dosage at which opioid dependency ensues (3 mg/kg/day; assuming a body weight of 400 g) (Pierce et al., 1992), but which does not disrupt pregnancy (Daly et al., 2012) rats were provided with a 0.2 ml/L solution of methadone, diluted in water. Solutions were provided to the rats in free-access water bottles, and access to other water sources was removed. Solutions were provided to the rats in free-access water bottles, and access to other water sources was removed. Dams were maintained on MTD throughout the postnatal period, so that pups were maintained on MTD through breast-milk.

2.2. Brainstem preparation

In accordance with methods approved by the Institutional Animal Care and Use Committee, neonate Sprague–Dawley rat pups (P0-P2) were anesthetized with isoflurane, and the sagittaly sectioned rat hindbrain preparation was isolated according to methods described elsewhere (Mellen, 2008; Mellen and Funk, 2013) to expose the VRC along its major axis, sectioned at the rostral margin of the facial nucleus (VIIn).

The preparation was then incubated in the medium-affinity Ca^{2+} indicator fluo 8 L AM (50 μg, $K_d = 1.86$ μM, AAT Bioquest), solubilized in 25 μL of the surfactant pluronic F-127 (2 g/10 ml DMSO; Invitrogen), and diluted in aCSF to a final concentration of 10 μM, and transferred to the recording chamber (2 ml bath volume; JG 23 W/HP; Warner Instruments, Hamden CT), mounted on an upright microscope (Axioskop 2 FS; Zeiss Instruments, Jena, DE). Throughout subsequent recordings, the preparation was perfused at 4 ml/min with aCSF warmed to 27 °C and aerated with a 95%–5% O_2-CO_2 gas mixture, which stabilized pH at 7.4.

2.3. Western blot analysis

Using the same anesthesia and surgery protocols described above, brainstems were rapidly isolated for protein preparation from P3, P6 and P10 control and MTD-exposed rat pups, flash-frozen with liquid nitrogen and stored at −80 °C. Protein samples were prepared in CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO) plus Complete Protease Inhibitors (Roche, Indianapolis, IN) at 4 °C. Equivalent total protein amounts were loaded onto 7% or 10% polyacrylamide gels (Bio-Rad, Hercules, CA) and then transferred to Protan BA83 Nitrocellulose Membranes (Midwest Scientific, Valley Park, MO) as previously described (Cai et al., 2012). Blots were probed and recognized with the following 1st and 2nd antibodies: rabbit anti-MBP (1:6000, Millipore), mouse anti-CNP (1:100, Millipore), mouse anti-phospho-NF200 (1:1000, Abcam), anti-β-actin (1:5000, Sigma-Aldrich, St. Louis, MO), and HRP-linked goat-anti-mouse, or -rabbit (Jackson Immunology, West Grove, PA). Signals were developed using chemiluminescence with ECL western blotting detection reagent (Pierce, Grand Island, NY) and exposed to film. The optical density (OD) of bands on Western blot was measured using ImageJ (Schneider et al., 2012). The ODs for specific protein were normalized over the ODs for β-actin, and these values were expressed as the ratio relative to the sham.

2.4. Pharmacological manipulation

In order to test for endogenous opioidergic modulation of respiratory networks, the mu-opioid antagonist naloxone (NAL) was bath applied. Stock solutions were prepared from 10 mM aliquots diluted in aCSF to a final concentration of 10 μM. Preparations were allowed to equilibrate for 5 min following drug wash-in before the second optical recording were carried out to detect NAL-induced changes in respiratory network activity.

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